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Comparison of Midgut Proteinases in *Bacillus thuringiensis*-Susceptible and -Resistant European Corn Borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae)

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The midgut proteinases from a *Bacillus thuringiensis*-susceptible (IA-S) and four laboratory-selected resistant strains (KS-SC, KS-NE, IA-1, and IA-3) of European corn borer (*Ostrinia nubilalis*) were characterized using three synthetic substrates, *N*α-benzoyl-L-arginine *p*-nitroanilide (BA*p*NA) for trypsin-like, *N*-succinyl-ala-ala-pro-phe *p*-nitroanilide (SAAPF*p*NA) for chymotrypsin-like, and *N*-succinyl-ala-ala-pro-leu *p*-nitroanilide (SAAPL*p*NA) for elastase-like proteinase activities. The hydrolyzing efficiency of trypsin-like proteinases, determined by V_{max}, decreased 35% in the KS-SC resistant strain compared with the susceptible strain. There were no significant differences in the Michaelis constant (K_m) among the five strains for the same substrate. When the purified *B. thuringiensis* Cry1Ab protoxin was used as the substrate, there was a detectable reduction in the hydrolysis of protoxin that was mediated by midgut proteinases from the KS-SC strain compared with the IA-S strain. Thus, the reduced trypsin-like proteinase activity appeared to lead to the reduced activation of the *B. thuringiensis* protoxins. This may confer or contribute to *B. thuringiensis* resistance in this strain. However, no significant difference was found in trypsin activity between the IA-S strain and the three other resistant strains (i.e., KS-NE, IA-1, and IA-3) and in chymotrypsin activity among all strains examined. These results suggest that other resistance mechanisms are responsible for the *B. thuringiensis* resistance in the KS-NE, IA-1, IA-2, and IA-3 strains of European corn borer.

Key Words: Bacillus thuringiensis; chymotrypsin; elastase; enzyme kinetics; European corn borer; insecticide resistance; proteinase; trypsin.

INTRODUCTION

Insect midgut proteinases have been demonstrated to play an important role in both the solubilization and the activation of *Bacillus thuringiensis* (*Bt*)[2] protoxins (1–12). Proteinases involved in protoxin activation are described as trypsin- or chymotrypsin-like proteinases in several insect species (3–8). Comparative studies on midgut proteinases from the *Bt*-susceptible and -resistant strains of the Indianmeal moth (*Plodia interpunctella*) suggested that altered protoxin activation by gut proteinases was

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[2] Abbreviations used: *Bt*, *Bacillus thuringiensis*; BA*p*NA, Nα-benzoyl-L-arginine *p*-nitroanilide; SAAPF*p*NA, *N*-succinyl-ala-ala-pro-phe *p*-nitroanilide; SAAPL*p*NA, *N*-succinyl-ala-ala-pro-leu *p*-nitroanilide; BCA, bicinchoninic acid solution; BSA, bovine serum albumin; ECB, European corn borer (*Ostrinia nubilalis*); SDS, sodium dodecyl sulfate; IA-S, *Bt*-susceptible strain of ECB initiated from eggs provided by the USDA Corn Insects Laboratory at Ames, Iowa; IA-1, IA-2, and IA-3, three *Bt*-resistant strains of ECB initiated from eggs provided by the USDA Corn Insects Laboratory and selected for resistance using a commercial formulation of *Bt* subsp. *kurstaki* (Dipel ES) under the laboratory conditions; KS-SC and KS-NE, two *Bt* (Dipel)-resistant strains derived from egg masses collected from corn fields near St. John in southcentral Kansas and near Manhattan in northeast Kansas, respectively, and selected for resistance using Dipel ES under the laboratory conditions; PAGE, polyacrylamide gel electrophoresis; Tris, Tris (hydroxymethyl)-aminomethane.

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involved in *Bt* resistance in several resistant strains of that insect (10–12). Alterations in the midgut enzymatic activity could change the *Bt* protoxin activation and/or toxin degradation and thus contribute to resistance to *Bt* toxins.

Moderate levels of laboratory-selected resistance to a commercial formulation of *Bt* have been achieved in several strains of European corn borer (ECB, *Ostrinia nubilalis*) (13). Resistance ratios of these strains ranged from 40- to 70-fold after 12–20 selected generations. Genetic studies suggested that the *Bt* resistance in a resistant (KS-SC) strain was incompletely dominant, and only one or a few major loci influenced this resistance (14). However, nothing is known about the mechanism(s) conferring resistance in these *Bt*-resistant strains of ECB.

The objective of this study was to investigate whether or not alterations of midgut proteinases were responsible for or contributed to *Bt* resistance in ECB. In this paper, we report (1) kinetic comparisons of the midgut proteinases from a *Bt*-susceptible and four -resistant strains of ECB using three synthetic proteinase substrates and (2) comparisons of *in vitro* hydrolysis of protoxin (Cry1Ab) by midgut proteinases from *Bt*-susceptible and -resistant strains of ECB.

MATERIALS AND METHODS

Insect Sources

The ECB strains were reared in our laboratory at Kansas State University, Manhattan, Kansas. Details about the origin, cultivation, selection, and bioassay of these strains have been described previously (13). Five resistant strains, KS-SC, KS-NE, IA-1, IA-2, and IA-3, were selected for 12–20 generations using a commercial formulation of *Bt* subsp. *kurstaki* Dipel ES (17,600 IU/mg; Abbott, North Chicago, IL), which was incorporated into an agar-based meridic diet. Dipel ES contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B endotoxins.

Chemicals and B. thuringiensis Toxin

Nα-benzoyl-L-arginine *p*-nitroanilide (BA*p*NA), *N*-succinyl-ala-ala-pro-phe *p*-nitroanilide (SAAPF*p*NA), *N*-succinyl-ala-ala-pro-leu *p*-nitroanilide (SAAPL*p*NA), bicinchoninic acid solution (BCA), and bovine serum albumin (BSA) were purchased from Sigma Chemical Company (St. Louis, MO). GelCode Coomassie blue stain reagent and Tris (hydroxymethyl)-aminomethane (Tris) were purchased from Pierce (Rockford, IL) and Bio-Rad Laboratories (Hercules, CA), respectively. Recombinant *Eschrichia coli* possessing the Cry1Ab gene from *Bt* subsp. *berliner* was kindly provided by Plant Genetic Systems (Gent, Belgium).

Extraction of Insect Midgut Proteinases

Midgut proteinases were obtained from ECB using the method described by Oppert *et al.* (10). Briefly, early fifth instars were chilled on ice for approx 30 min, and the posterior and anterior ends of the larvae were removed. Midguts were excised with forceps and submersed immediately in ice-cold 200 mM Tris–HCl buffer (pH 8.0) containing 20 mM $CaCl_2$. Twenty midguts were placed in each 1.5-ml microcentrifuge tube containing 0.5 ml of the same buffer and homogenized in the microcentrifuge tube on ice using a plastic pestle. After the homogenates were centrifuged at 15,000*g* for 5 min, the supernatants were aliquoted at 50 μl and stored in 0.5-ml microcentrifuge tubes at -20°C until use.

Assays and Kinetic Analysis of the Midgut Proteinases

Three synthetic proteinase substrates, BA*p*NA, SAAPF*p*NA, and SAAPL*p*NA, were used to determine the trypsin-like, chymotrypsin-like, and elastase-like proteinase activities, respectively (15–17). To evaluate the effects of pH condition on the proteinase activity, the midgut extracts from the Dipel-susceptible strain (i.e., IA-S) were assayed at a concentration of 1 mg of substrate/ml (i.e., 2.3 mM for BA*p*NA, 1.6 mM for SAAPF*p*NA, and 1.7 mM for SAAPL*p*NA) in 200 mM Tris–HCl buffer containing 20 mM $CaCl_2$. Ten different pH conditions, ranging from 5.5 to 10, were tested. Enzyme activity was determined at room temperature using a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA) at 405

nm. Our preliminary assays indicated that nonenzymatic hydrolysis of these *p*-nitroanilide substrates was negligible under the given assay conditions (even at pH 10). Therefore, no control was incorporated into our assays to account for any nonenzymatic hydrolysis of the substrates.

For enzyme kinetic analysis, each substrate was dissolved in 200 mM Tris–HCl buffer (pH 9.0) containing 20 mM $CaCl_2$ at 11 concentrations with a range of 1 μg to 1 mg of substrate/ml (i.e., 2.3 μM–2.3 mM for BA*p*NA, 1.6 μM–1.6 mM for SAAPF*p*NA, and 1.7 μM–1.7 mM for SAAPL*p*NA). After the proteinase extract was diluted 100-fold with the same buffer, the reaction was initiated by mixing 100 μl of the diluted enzyme preparation (0.04 midgut equivalent) and 100 μl of substrate solution in a 96-well microplate. The initial velocity was determined immediately at room temperature in the microplate reader at 405 nm. Initial velocities obtained from the first five substrate concentrations at high region were used for kinetic analysis of trypsin-like proteinases and eight concentrations for both chymotrypsin- and elastase-like proteinases.

Proteinase activities for all three substrates were calculated based on the molar extinction coefficient of 8800 for the reaction product *p*-nitroaniline (18). Michaelis constant (K_m) and maximal velocity (V_{max}) values for each substrate were determined by Hanes transformations (19). Analysis of variance (ANOVA) (20) was used to compare K_m and V_{max} among different ECB strains for each substrate.

Protoxin Purification and Protoxin Hydrolysis by Midgut Proteinases

Recombinant *E. coli* possessing Cry1Ab genes from *Bt* subsp. *berliner* were grown on LB media at 37°C, and recombinant Cry1Ab protoxin was purified as previously described (21,22). For analysis of *Bt* protoxin hydrolysis/activation by proteinases, 20-μl reaction mixtures containing 0.3 μg ECB midgut extracts and 10 μg purified Cry1Ab protoxin in 200 mM Tri–HCl buffer containing 10 mM $CaCl_2$ (pH 8.0) were incubated at 37°C for various periods of time. Because the incubation time of 15 s appeared to be optimal for the protoxin hydrolysis under the given conditions, this incubation time was used in our sequential studies. Reactions were stopped by incubating them in a water bath at 95°C for 5 min and then cooling them to room temperature. After 20 μl of reducing sample buffer was added to each reaction, samples were again heated at 95°C for 5 min. Eighteen-microliter aliquots were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE, 23), using 4–12% precast gels (Novex, San Diego, CA). Gels were stained with GelCode Coomassie blue stain reagent.

Determination of Protein Concentration

Protein concentration of proteinase preparations was determined according to Smith *et al.* (24) using bovine serum albumin as a standard. The measurement was performed with the microplate reader at 560 nm.

RESULTS

Effects of pH and Substrate Concentration on the Midgut Proteinase Activity

Midgut proteinases from all five strains of ECB hydrolyzed the trypsin (BA*p*NA), chymotrypsin (SAAPF*p*NA), and elastase (SAAPL*p*NA) substrates. For all three substrates, the enzyme activity was pH dependent and increased as pH increased to 10, the highest pH condition examined in this study (Fig.1). Apparently, the optimal pH for the midgut proteinases from the ECB larvae could be 10 or higher.

The trypsin-like proteinase activity in the KS-SC resistant strain was significantly lower than that of all other strains at BA*p*NA concentrations of 0.29–2.3 mM (Fig. 2). Compared to the susceptible strain (IA-S), the enzyme activity was reduced approx 30–35% at this substrate concentration range. Generally, there was no significant difference in the trypsin-like proteinase activity between the resistant (IA-1) and the susceptible (IA-S) strains across the tested substrate concentrations except at the concentration of

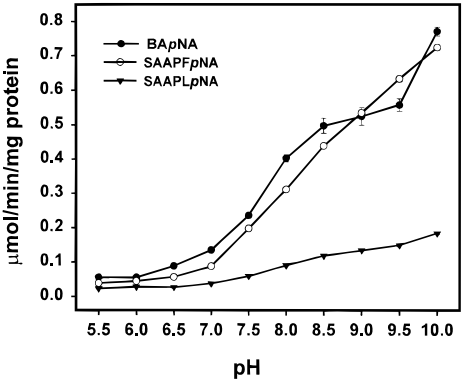

FIG. 1. Midgut proteinase activity in a *B. thuringiensis*-susceptible strain (IA-S) of European corn borer larvae for three substrates at different pH conditions. Each point represents the mean of three assays ($n = 3$), each with duplicated determinations. Vertical bars indicate standard errors of the mean.

0.14 mM. At this concentration, significantly lower trypsin-like proteinase activity was found in the resistant strain (IA-1) than in the susceptible strain (IA-S). However, no significant difference in the trypsin activity was observed between the resistant (IA-3) and the susceptible (IA-S) strains across all substrate concentrations examined in this study.

In contrast, relatively high trypsin-like proteinase activity was observed for the KS-NE resistant strain at substrate concentrations ranging from 0.29 to 2.3 mM. There were no significant differences among the five strains in trypsin-like proteinase activity at the lower substrate concentrations. Although the IA-3 resistant strain had a relatively higher chymotrypsin-like proteinase activity across many of the tested substrate concentrations, overall difference among the five ECB strains was small (Fig. 3). The elastase-like proteinase activity was generally lower in the KS-SC strain and higher in the other resistant strains of ECB than in the control strain (IA-S) but this enzyme activity in all these strains was significantly lower than the trypsin- and chymotrypsin-like proteinase activities, with relatively little variability among the different strains (Fig. 4).

Kinetic Analysis of Midgut Proteinases

Kinetic studies were conducted to determine the affinity of the midgut proteinases for the three substrates. There were no significant differences in the K_m values among the five strains of ECB for the same substrate. However, the hydrolyzing efficiency, measured by V_{max}, of

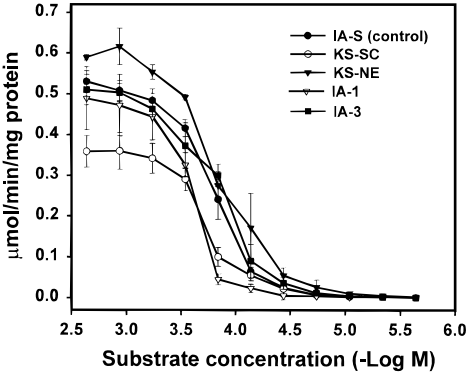

FIG. 2. Midgut proteinase activities of a *B. thuringiensis*-susceptible (i.e., control) and four -resistant strains (i.e., KS-SC, KS-NE, IA-1, and IA-3) of European corn borer larvae at different concentrations of trypsin synthetic substrate (BA*p*NA). Each point represents the mean of three assays ($n = 3$), each with duplicated determinations. Vertical bars indicate standard errors of the mean.

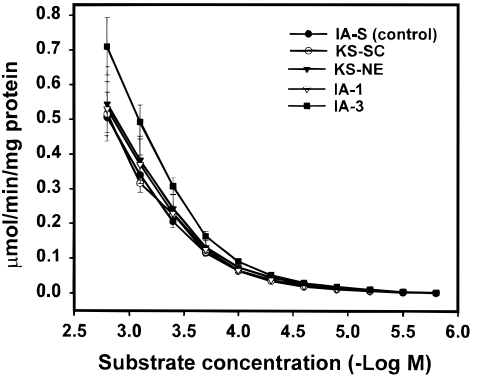

FIG. 3. Midgut proteinase activities of a *B. thuringiensis*-susceptible (i.e., control) and four -resistant strains (i.e., KS-SC, KS-NE, IA-1, and IA-3) of European corn borer larvae at different concentrations of chymotrypsin synthetic substrate (SAAPF*p*NA). Each point represents the mean of three assays ($n = 3$), each with duplicated determinations. Vertical bars indicate standard errors of the mean.

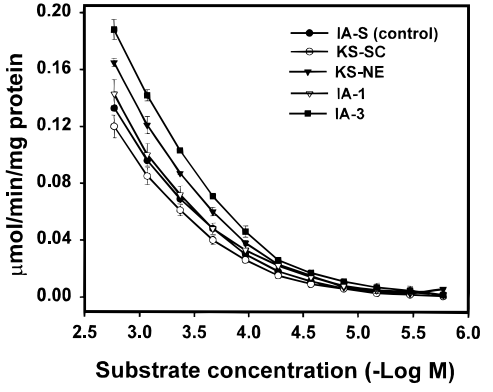

FIG. 4. Midgut proteinase activities of a *B. thuringiensis*-susceptible (i.e., control) and four -resistant strains (i.e., KS-SC, KS-NE, IA-1, and IA-3) of European corn borer larvae at different concentrations of elastase synthetic substrate (SAAPL*p*NA). Each point represents the mean of three assays ($n = 3$), each with duplicated determinations. Vertical bars indicate standard errors of the mean.

trypsin-like proteinases was significantly lower in the KS-SC resistant strain than in the other strains, except for the IA-1 resistant strain (Table 1). The hydrolyzing efficiency of trypsin-like proteinases from the KS-SC resistant strain decreased 35% compared to the susceptible strain (IA-S). However, there were no significant differences in the hydrolyzing efficiency of the trypsin-like proteinases among the other four strains. In contrast, the hydrolyzing efficiency of elastase-like proteinases was significantly higher in the IA-3 resistant strain compared to the susceptible and the other resistant strains, except for the KS-NE strain. There were no significant differences in hydrolyzing efficiency for chymotrypsin-like proteinases among the five insect strains (Table 1).

Comparison of Bt Protoxin Hydrolysis by Midgut Proteinases

There was a detectable difference in the hydrolysis of the protoxin Cry1Ab mediated by midgut proteinases of the KS-SC strain compared with that of the susceptible strain (IA-S) of ECB (Fig. 5). After 15 s of reaction, the amount of protoxin (ca. 126-kDa protein) recovered on the SDS–PAGE was relatively higher in the KS-SC strain than in the IA-S and other resistant strains (KS-NE and IA-2), suggesting that the hydrolytic process of the *Bt* protoxin in the KS-SC strain was reduced. However, the other resistant strains did not show significant

TABLE 1
Kinetic Parameters of Midgut Proteinases from a *Bt*-Susceptible (IA-S) and Four *Bt*-Resistant Strains of ECB[a]

Substrate	Strain	V_{max} (μmol/min/mg)	K_m (mM)	V_{max}/K_m[b]
BA*p*NA	IA-S	0.57 ± 0.02 A	0.14 ± 0.02 A	4.07
	KS-SC	0.37 ± 0.04 B	0.13 ± 0.02 A	2.85
	KS-NE	0.63 ± 0.004 A	0.11 ± 0.03 A	5.73
	IA-1	0.55 ± 0.08 A	0.24 ± 0.09 A	2.29
	IA-3	0.54 ± 0.04 A	0.11 ± 0.02 A	4.91
SAAPF*p*NA	IA-S	0.88 ± 0.09 A	1.25 ± 0.07 A	0.70
	KS-SC	0.97 ± 0.16 A	1.36 ± 0.15 A	0.71
	KS-NE	0.89 ± 0.22 A	1.00 ± 0.12 A	0.89
	IA-1	0.97 ± 0.20 A	1.28 ± 0.24 A	0.76
	IA-3	1.22 ± 0.19 A	1.17 ± 0.11 A	1.04
SAAPL*p*NA	IA-S	0.16 ± 0.002 A	0.48 ± 0.01 A	0.33
	KS-SC	0.15 ± 0.01 A	0.54 ± 0.02 A	0.28
	KS-NE	0.20 ± 0.01 AB	0.47 ± 0.06 A	0.43
	IA-1	0.17 ± 0.01 A	0.48 ± 0.05 A	0.35
	IA-3	0.23 ± 0.01 B	0.44 ± 0.05 A	0.52

[a] Results are the mean ± SEM of three assays ($n = 3$), each with duplicated determinations. Means within columns followed by the same letter are not significantly different ($P > 0.5$, LSD multiple range test) (20).

[b] Substrate specificity constant.

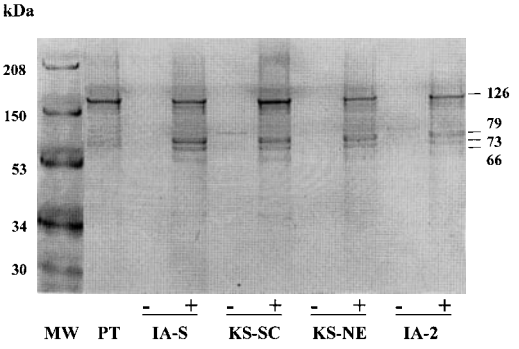

FIG. 5. SDS–PAGE analysis of protoxin (Cry1Ab) hydrolysis by midgut proteinases from the *Bt* (Dipel ES)-susceptible (IA-S) and -resistant (KS-SC, KS-NE, and IA-2) strains of ECB. MW, molecular weight markers; PT, protoxin only; IA-S, KS-SC, KS-NE, and IA-2, ECB midgut extract proteins from each strain incubated without (−) or with purified Cry1Ab protoxin (+) followed by SDS–PAGE analysis.

differences in the hydrolysis of *Bt* protoxin (Fig. 5).

DISCUSSION

In the spruce budworm (*Choristoneura fumiferana*), partially purified gut proteinases were used to characterize their pH-dependent maximal hydrolysis of synthetic amino acid esters, amide substrates, and denatured proteins (8). Optimal pH observed for these proteinases were from 9.0 to 11.5. Houseman *et al.* (25, 26) also reported that the total proteolysis of ECB larval gut enzymes, measured using azocasein, exhibited maximal activity at pH 10.0 or higher. These results were later confirmed by Bernardi *et al.* using purified trypsin-like enzyme from ECB (27). In this study, we found that the midgut proteinase activity against BA*p*NA, SAAPF-*p*NA, and SAAPL*p*NA increased as pH increased within the tested pH range of 5.5–10. Our results suggested that the maximal hydrolysis of these three synthetic substrates was at a pH 10 or higher. This finding was consistent with other published studies with respect to the optimal pH and consistent with the alkaline environment of the lepidopteran midgut (25).

Our study also demonstrated that the midgut proteinases varied in both substrate specificity and enzyme activity in ECB. Compared to the susceptible strain (IA-S), the V_{max} for trypsin-like proteinase activity was reduced 35% in the KS-SC resistant strain. When the concentration of the trypsin-like proteinase substrate was reduced to 0.14 mM in the reaction mixture, an approx 59 and 81% reduction in trypsin-like proteinase activity was observed in the KS-SC and IA-1 strains, respectively (Fig. 2). However, the degree of reduction of trypsin-like proteinase activity in the IA-1 strain was not consistent with the enzyme kinetic data that showed no significant difference between the IA-S and the IA-1 strains. In contrast, our kinetic analysis of the trypsin-like proteinase activity from a substrate concentration range of 0.14–2.3 mM for the KS-SC strain corresponded to the enzyme activity.

Several studies have demonstrated that enzymatic alterations of midgut proteinases can play an important role in *Bt* resistance via the reduction of the protoxin activation by insect midgut proteinases (2, 10–12). This is particularly true for trypsins, since numerous studies suggested that trypsins were important proteinases responsible for activation of *Bt* protoxins in the insect midgut (1–12). In the KS-SC *Bt*-resistant strain of ECB, results from our proteinase kinetic and *Bt* protoxin activation studies complemented each other. Thus, it appears that reduced hydrolysis of *Bt* protoxins by midgut trypsin-like proteinases may contribute to *Bt*, particularly Cry1Ab, resistance in the KS-SC strain. These results support the hypothesis that reduced activation of *Bt* protoxins by insect gut proteinases was responsible for or contributed to *Bt* resistance in *Culex quinquefasciatus* (2) and the Indianmeal moth (10–12).

However, we did not find significant differences in midgut proteinase activity and *Bt* protoxin activation between the *Bt*-susceptible (IA-S) and the other -resistant strains (KS-NE, IA-1, IA-2, and IA-3). It is likely that other resistance mechanisms are present in these resistant strains. In several insect species, the midgut brush border membrane receptors have been found to play an important role in the mode of action of *Bt* and resistance to *Bt* toxins (28–32). For example, the

CryIAb resistance in the Indianmeal moth was attributed to a reduced binding affinity to the midgut brush border membrane receptors (33). The Cry1Ab resistance of the diamondback month (*Plutella xylostella*) was associated with a modified interaction of the toxin with its midgut binding site (34). Changes in the midgut binding sites have also been found in resistant tobacco budworm (*Heliothis virescens*) but other mechanisms may also be involved in the resistance (35, 36). In the ECB, further studies are needed to clarify which trypsin(s) are associated with *Bt* resistance in the KS-SC strain but, more importantly, other resistance mechanisms, such as modifications in the binding of *Bt* toxins to the midgut brush border membrane, should also be examined. It is possible that altered binding between the *Bt* toxins and the midgut binding receptors may contribute to *Bt* resistance in the KS-SC strain as well as in the KS-NE, IA-1, IA-2, and IA-3 strains.

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